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Disintegrins from Snake Venoms and their Applications in Cancer Research and Therapy

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Abstract: Integrins regulate diverse functions in cancer pathology and in tumor cell development and contribute to important processes such as cell shape, survival, proliferation, transcription, angiogenesis, migration, and invasion. A number of snake venom proteins have the ability to interact with integrins. Among these are the disintegrins, a family of small, non-enzymatic, and cysteine-rich proteins found in the venom of numerous snake families. The venom proteins may have a potential role in terms of novel therapeutic leads for cancer treatment. Disintegrin can target specific integrins and as such it is conceivable that they could interfere in important processes involved in carcinogenesis, tumor growth, invasion and migration. Herein we present a survey of studies involving the use of snake venom disintegrins for cancer detection and treatment. The aim of this review is to highlight the relationship of integrins with cancer and to present examples as to how certain disintegrins can detect and affect biological processes related to cancer. This in turn will illustrate the great potential of these molecules for cancer research. Furthermore, we also outline several new approaches being created to address problems commonly associated with the clinical application of peptide-based drugs such as instability, immunogenicity, and availability.

Keywords: Antitumor, carcinogenesis, cell death, integrins, metastasis, snake venoms, tumor promotion.

1. INTRODUCTION

Globocan estimated that in 2012, there were 14.1 million new cases of cancer diagnosed, 8.2 million deaths from cancer, and 32.6 million people living with cancer worldwide. Nevertheless, despite all the advancements in the development of new approaches to improve cancer screening, diagnosis, and treatment, cancer still is responsible for significant death and morbidity around the globe [1].

Historically, venomous snakes have fascinated mankind due to the dramatic effects envenomation has on their prey and/or enemies and subsequently the pharmacological implications associated with their venoms. However, in spite of their toxicological effects, several snake venom proteins (e.g. disintegrins, phospholipases A2, metalloproteinases, and L-amino acid oxidases) and peptides (e.g. bradykinin potentiators, natriuretic, and analgesic peptides) have demonstrated potential to provide practical applications such as pharmaceutical agents, including areas of cancer treatment and diagnosis [2-4]. Among components that comprise the complex mixture of biomolecules of which snake venoms are made, disintegrins might interfere in important cancer related processes [5]. This review is an effort to illustrate the relationship of integrins with cancer, and the effect of disintegrins in relevant biological processes. In addition, we will highlight the disintegrins that may be of use in the diagnosis, treatment, and evaluation of cancer.

1.1. Cancer Development

Normal growth of tissue occurs due to a delicate balance between pro and anti-apoptotic pathways, which control the cell metabolism and tissue homeostasis. Carcinogenesis begins when this balance is altered in favor of prolonged cell survival following molecular alterations, which lead to the cell acquiring a malignant phenotype [6]. Those changes can be divided into three general stages: initiation, promotion and progression, although these may not always exactly represent the step-wise genotypic and phenotypic changes encompassed in carcinogenesis [7].

Initiation involves mutational events and results in little or no observable changes in the cellular or tissue morphology, but confers a permanent increase in susceptibility to cancer formation. Promotion is defined as a process by which the initiated cell clonally expands, resulting in formation of a non-malignant visible tumor [8]. From this stage, angiogenesis comprises a key role in tumor development, since it is essential in providing the metastatic tumor with the blood's nutrients and oxygen, along with being an effective way to remove waste products [9].

In progression, tumors become malignant through the accumulation of additional genetic and epigenetic alterations.

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In this phase, tumors also require some additional tissue disruption and cellular adaptations to harsh microenvironmental conditions, including hypoxia and acidosis [10]. Metastasis is described as a multistage process in tumor progression, in which malignant cells spread from the tumor's origin to colonize distant organs. It is known that this complex process involves capabilities inherent to the tumor cells, as well as components of the stromal microenvironment, such as the formation of a pre-metastatic niche [11, 12].

The classical simplification of dissemination of tumor cells via the blood stream and metastatic colonization at distal sites can be described by several basic steps, including migration, invasion and intravasation, survival in the circulation, adhesion to the vasculature wall, extravasation, and finally, proliferation in the host tissue [13]. Integrins are involved in all stages of this process and they are essential not only by mediating adhesion to the ECM, but also by regulating intracellular signaling pathways that control, for example, cytoskeletal organization and cell survival [14].

1.2. Integrins

Much of the classic literature regarding cancer has given integrins a crucial role in tumorigenesis, affecting tumor initiation, promotion, and progression [15, 16]. Integrins are a family of heterodimeric transmembrane proteins formed by the noncovalent association of α- and β-subunits, comprised of approximately 18 and 8 subunits types respectively, forming approximately 24 different integrins (Fig. **1**) [17]. Integrins are functionally capable of promoting cell-ECM interactions as well as intercellular signaling in addition to connect the ECM to the cell cytoskeleton [5]. Extracellular divalent cations, such as Ca^{2+} and Mg^{2+}, may influence the specificity and affinity of integrins when binding to their ligands [18].

Furthermore, integrins participate in many signaling processes that affect cellular functions such as cytoskeleton organization, transduction of intracellular signals, adhesion, growth, survival, differentiation, development, and apoptosis. They are also related to biological functions such as tissue repair, immune responses and leukocyte traffic, as well as many human disorders including some genetic and autoimmune diseases and others such as cancer [5].

1.3. Integrins and Cancer

Integrins regulate diverse functions in tumor cells; for example, it relays molecular cues from the cellular environment that influence cell shape, survival, proliferation, transcription, and migration (Fig. **2**) [19]. Many integrins expressed by epithelial cells (including α6β4, α6β1, αvβ5, α2β1, and α3β1) have altered expression levels on cancer cells of epithelial origin. Normally these integrins mediate epithelial cell adhesion to the basement membrane, but in tumor cells they may contribute to proliferation, migration, invasion, and survival [20]. Furthermore, integrin expression can vary considerably between normal and tumor tissue. For example, integrins αvβ3, α5β1, and αvβ6 are usually expressed at low or undetectable levels in adult epithelia, but can be highly up regulated in some tumors [21]. Conversely, α2β1 integrin expression is diminished or absent in adenocarcinoma of the breast and in other epithelial malignancies [22], and its re-expression in breast cancer cells reversed some of the malignant properties of those cells, suggesting that it could function as a tumor suppressor [23].

In fibroblasts, integrin α1β1 down-regulates collagen and reactive oxygen species (ROS) production and promotes cell proliferation [24]. Studies have also identified the critical and collaborative function of α1β1 and α2β1 integrins in supporting VEGF signal transduction and endothelial cell migration [25]. For instance, cells expressing α2β1 preferentially adhere to collagen IV, as well as laminin. The α subunit is an important determinant for ligand recognition and for binding of these integrins [25, 26]. Similarly, α1β1 and α2β1 integrins, through binding to collagen, induce VEGF and participate of VEGF-driven angiogenesis [24] and cell migration [25].

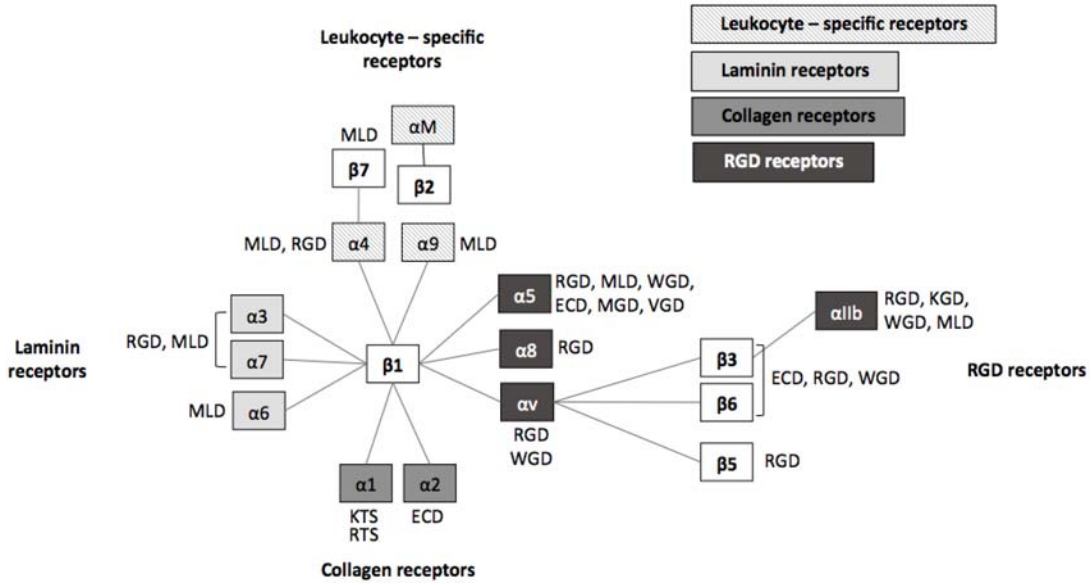

Fig. (1). Diagram with the human integrins and the active site present in snake venom disintegrins (consisting of a triad of amino acids) able to interact with them. The relation of integrins with specific receptors is provided by alpha chains and is represented in different colors.

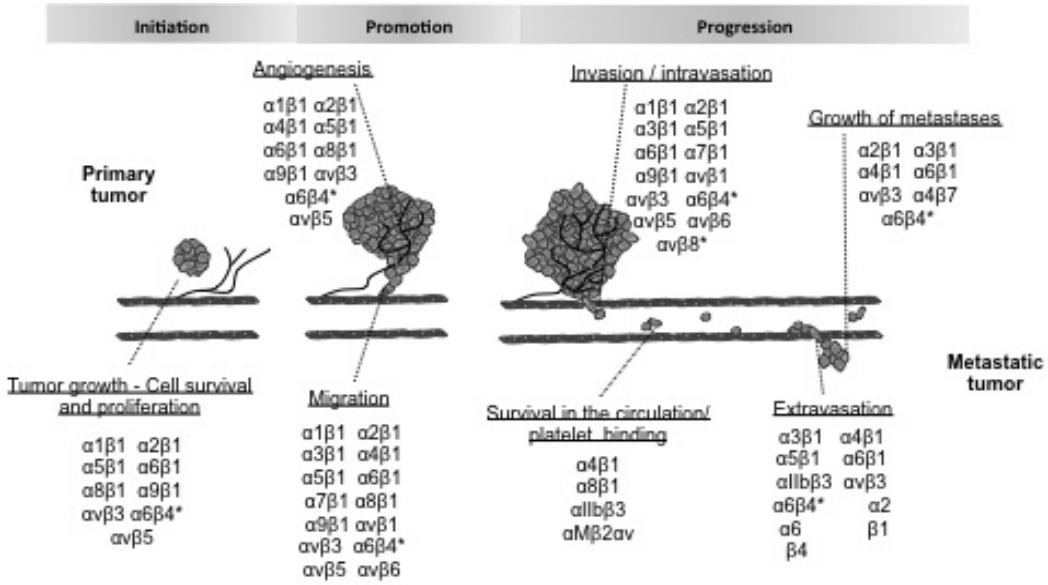

Fig. (2). Integrins involved in each step of tumor development and progression. *No disintegrin described was able to interact with this integrin.

The activity of α3β1, on the other hand, seems somewhat more complex, since it interacts with multiple ligands including fibronectin, collagen, entacin/nidogen, epiligrin, thrombospondin-1, and many laminin isoforms [26]. The interaction of α3β1 with laminin-5 has been demonstrated to promote the migration and invasion of malignant glioma and melanoma cells [27]. Moreover, α3β1 integrin plays an important function of cell arrest in the pulmonary vasculature and early colony formation [28].

Studies with adhesion-blocking reagents and knockout mice suggest that the α4β1 integrin has a crucial role in angiogenesis, since it is necessary for an interaction of endothelial cells with VCAM1 on pericytes, resulting in vessel stabilization [29, 30]. It also regulates appropriate Rac activation to drive leukocyte migration, thus developing a role in the survival of cancer cells in the bloodstream and extravasation [31]. *In vitro* experiments have demonstrated that both osteo- and rhabdo-myosarcoma cell lines adhere to endothelium via the α4β1-vascular cell adhesion molecule 1 (VCAM-1) pathway [32], [33], while *in vivo* evidences corroborate the role of α4β1-VCAM-1 interaction in sarcoma adhesion and subsequent cell extravasation [34]. Furthermore, α4β1 integrin is essential for the pre-metastatic niche formation, since it participates in the migration of hematopoietic cells within the bone marrow [35].

The α5β1 integrin is implicated in several cellular activities including cell proliferation, differentiation, and migration due to its interaction with fibronectin and its contribution on cell–cell cohesion indirectly through binding to intercellular ECM components [26, 36]. Integrin α5β1 is important for tumor growth both *in vitro* and *in vivo* [37] and it is usually expressed at low or undetectable levels in most adult epithelia, but can be highly upregulated in some tumors and on the luminal surface of tumor vessels [38]. Therefore, studies with adhesion-blocking reagents and knockout mice have identified its crucial role in angiogenesis [30, 39]. It also forms a complex with the protein Rab25 to support the formation of long pseudopodial extensions, which may promote cell migration and invasion in 3D contexts [40]. Finally, α5β1 integrin participates in cell extravasation through increasing the production of active MMPs and inducing anoikis-resistance, a pre-requisite for tumor cells to intravasate into the circulation and extravasate into distant organs, in addition to contributing to cell survival [41].

Integrin α6β1 is a laminin receptor. It has been reported that α6 integrin mediate neutrophil migration through the perivascular basement membrane (PBM) [42]. In addition, α6 integrin is found to be overexpressed in human esophageal carcinomas, suggesting an important role in esophageal tumor invasion [43]. Integrin α6β1 also contributes to cell–cell cohesion [44], promotes prostate tumor cell survival [45], angiogenesis [30], and migration [46].

Conversely, integrin α7β1 is the dominant laminin-binding integrin in muscle and plays diverse roles during the different stages of development [47]. This integrin is not expressed in human melanocytes, but it is upregulated in some human melanoma cells. Studies suggest that it is related with motility and invasion of cancer cells [48].

Integrin α8β1 binds several ligands including fibronectin, vitronectin, tenascin-C, osteopontin (OPN), and nephronectin [26]. As such, this integrin can promote a variety of biological functions including attachment, cell spreading, and neurite outgrowth related to fibronectin [49], tumor growth, metastasis, tumor angiogenesis, and inhibition of immune surveillance due to tenascin-C which, incidentally, is absent or greatly reduced in most adult tissues, but increases markedly in cancer development [50].

Integrin α9β1 interacts with many ligands such as angiostatin, a fragment of plasmin (plasminogen), tenascin-C, osteopontin, certain ADAM proteins, VCAM-1, tissue-type transglutaminase (tTG), factor XIII, and Von Willebrand factor (VWF). It plays a central role in inflammatory responses and in metastasis [26], might promote carcinoma growth [51],

and participate in angiogenesis [30]. Furthermore, it appears to induce a functionally relevant epithelial–mesenchymal transition (EMT) phenotype in lung cancer cells [51].

Integrin αvβ1 binds mainly to fibronectin, vitronectin, fibrinogen, and OPN [52]. Studies demonstrated its contribution in squamous cell carcinoma migration [53] and invasion through stimulating MMP2 expression [54].

The β2 integrins subfamily, including αMβ2, are immunologically restricted to leukocytes and typically have other cell surface molecules as their ligands. αMβ2, for example, recognizes fibrinogen, ICAMs, iC3b, and the factor-Xa [26]. Studies showed that this integrin regulates Rac activation to drive leukocyte migration, thus playing a role in cell survival in the bloodstream [31]. Moreover, by binding to fibrin (fibrinogen) αMβ2 promotes early inflammatory events in colitis, contributing to adenoma formation and growth [55].

Integrin αvβ3 has a broad distribution and is one of the more promiscuous receptors, capable of binding to a large number of ECM protein ligands including vitronectin, fibrinogen, fibronectin, and thrombospodin, as well as to other proteins such as VWF, fibroblast growth factor receptor-2 (FGFR2), MMP-2 and certain ADAM proteins [56-59]. The interaction of αVβ3 with its ligands plays a crucial role in tumor initiation, promotion and progression. For example, in endothelial cells, it cross talks with fibroblast growth factor receptor FGFR and inhibits the intrinsic apoptosis pathway [60]. Moreover, αvβ3 integrin mediates cell survival [61], angiogenesis [62] and cell migration [15, 63]. Integrin αvβ3 is also related with the survival of the metastatic cells in the bloodstream [64] and in the metastatic colonization of bone and lung through its interaction with bone matrix proteins such as osteopontin [65].

Another well-known and well-characterized integrin is αIIbβ3. Found on platelets and megakaryocytes, it plays an essential role in hemostasis and binds to collagen, fibronectin, vitronectin, fibrinogen, VWF, and TSP. As well as αvβ3, α IIbβ3 protects cells in the bloodstream, facilitating tumor cell arrest in the vasculature and leading to metastasis to various sites, including the bone marrow [13, 66].

Integrin α6β4, on the other hand, may be vital to tumor formation and they cooperate to induce spontaneous mammary tumor formation and tumor cell invasion [67]. Furthermore, integrins such as αvβ3 and α6β4 were reported to be involved in cell migration, promotion and movement [15, 36, 63]. Integrin α6β4 is an essential integrin for the organization and maintenance of epithelial structure that has a pivotal role in the biology of invasive carcinoma [68]. Evidence also shows that α6β4 integrin binds to chloride channel calcium-activated (CLCA), a Ca^{2+} sensitive channel expressed by pulmonary endothelial cells, and allows cancer cells to arrest in the microvascular bed of the lung, promoting their intravascular growth [69]. α4β7 and α6β4 integrins are also prominently expressed within the metastatic niche and contribute to breast cancer metastasis to the lungs by binding to the human calcium-activated chloride channel regulator 2 (CLCA2) expressed on pulmonary endothelial cells [70].

Clinically, αvβ5 is expressed on most ovarian cancers [71] and its expression is correlated with neuroblastoma aggressiveness [72]. Integrin αvβ5 binds to vitronectin and functions together with VEGF receptor 2 (VEGFR2) to prevent the extrinsic apoptosis pathway [73, 74]. Besides, it also participates in cell survival through several mechanisms, such as increasing expression of BCL-2 [61] or flice-like inhibitory protein (FLIP) [75], activating the PI3K–AKT pathway [76], NF-κB signaling [77], and/or inactivating p53 [78]. Integrin αvβ5 participates in angiogenesis [79] and besides its role in tumor growth and angiogenesis, αvβ5 has been found to be functionally involved in cell migration *in vitro* and metastasis *in vivo* [80, 81].

The integrin αvβ6 interacts with a large number of ligands including fibronectin, vitronectin, tenascin, and tissue growth factor-β (TGF- β). It also mediates the production and regulation of MMP-9 and MMP-3 [82]. In addition to that, integrin αvβ6 facilitates normal and transformed cell migration through interstitial matrices with a direct effect on the growth of tumor cells and it has a TGF-β-dependent effect on the invasion of carcinoma cells [83]. Moreover, it might promote epithelial-to-mesenchymal transition, thus contributing with a broad number of modulatory mechanisms that affect numerous biological functions, including cell proliferation, ECM synthesis and degradation, and cell migration [84].

Integrin α4β7, similarly to α4β1, is mostly expressed on leukocytes and mediate cell-cell and cell-ECM adhesion. Additionally, it is related with proliferation in the target tissue [26], being abundantly expressed in the metastatic niche and contributing to breast cancer metastasis to the lungs by binding to the human CLCA2 expressed on pulmonary endothelial cells [70].

1.4. Snake Disintegrins

Disintegrins are a family of small, non-enzymatic, cysteine-rich proteins found in the venoms of Viperidae, Crotalidae, Atractaspididae, Elapidae, and Colubridae snake families [85]. The first disintegrin isolated from snake venom was trigramin, a single-chain, cysteine-rich peptide purified from the venom of the *Trimeresurus gramineus* [86]. It contains the RGD sequence and inhibits the adhesion of human melanoma cells to fibronectin and fibrinogen [87]. Additionally, *in vivo* co-administration with cancer cells markedly inhibited tumor growth and bone destruction [88]. Since then, several new molecules have been identified and functionally and structurally characterized, giving rise to extensive possibilities for applications. These proteins have been demonstrated to have the capacity to interact with specific integrins and to inhibit their activity. They can be classified structurally or functionally according to the recognition motif capable of specifically binding to integrins (Fig. **1**).

1.4.1. Structural Classification

Typically, disintegrins are derived from proteolytically processed precursors, named snake venom metalloproteases (SVMPs), which are in turn, phylogenetically related with ADAMs (a disintegrin and metalloprotease) [89]. However, it has also been shown they may occur as the result of synthesis from short-coding mRNAs, based on their presence in the cDNA library of venom glands without a metalloproteinase (MP) domain [90]. SVMPs are classified according to

their multi-domain structure into P-I, P-II and P-III classes. Members of the P-I class are comprised of a MP domain. Proteins belonging to P-II class have a MP and a disintegrin-like domain, and P-III class proteins have a cysteine-rich domain following the disintegrin region and, in some cases, a lectin domain. These two last classes (P-II and P-III) can be subdivided according to the proteolytic processing of their domains and their ability to form dimeric structures [91].

Depending on the type and processing, SVMP originates different disintegrins and can be structurally divided in monomers, homodimers or heterodimers. Monomeric disintegrins are derived from processing of P-IIa class of SVMPs. They are usually low molecular weight (5-8 kDa) and contain the RGD sequence motif. This class of disintegrin is quite common in snake venoms and has been demonstrated to play a critical role in protein interactions with cell-surface integrins. Homodimeric disintegrins are derived from PIId class. Heterodimeric disintegrins are processed from PIIe SVMP, and some of them appear to be formed by one subunit, resulting from this processing a and another from a translated gene product, representing the disintegrin domain alone [91, 92].

PIII SVMPs, in turn, give rise to the disintegrin-like proteins (lack the RGD motif), formed by covalently linked-disintegrin-like and Cys-rich domain, with molecular masses around 30 kDa [91, 92]. Interestingly, disintegrins-like domains alone have not been isolated in a processed form from venom. They were found only as a biologically active protein containing the cysteine- rich domain, such as the Jararhagin-C from *Bothrops jararaca* [93] and catrocollastatin-C from *Crotalus atrox* venom [94]. This suggests that structurally those domains are very strongly associated, inhibiting further processing.

1.4.2. Functional Classification

The functional classification of disintegrins depends on their ability to interact with specific integrins, which is determined by the presence of a particular integrin-binding motif localized in the hairpin loop. Three functional classes containing RGD, MLD or R/KTS motifs have been identified [92]. RGD-disintegrins constitute the largest and most investigated family of disintegrins, and they inhibit the physiological functions of integrins α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, αvβ1, αvβ3, αIIbβ3, and αvβ5. The majority of identified RGD-disintegrins are monomeric, although there are some RGD subunits belonging to dimers and, in these cases, the second subunit may display another motif [95, 96].

Natural variations of the arginine in the RGD motif may result in moderation or abolishment of biological activity. For example, in a group of synthetic peptides, substitutions of an arginine in a RGD motif by tryptophan increased inhibitory activity toward certain integrins. In another case, alanine substitution of the aspartic acid in a WGD motif decreased its inhibitory ability, whereas this same positional substitution in a RGD motif almost completely abolished the activity of the peptides [97].

On the other hand, the MLD motif is found only in heterodimeric disintegrins and mediates the binding to integrins α3β1, α4β1, α5β1, α6β1, α7β1, α9β1, αIIbβ3, and α4β7. KTS or RTS motifs in the active site, in turn, selectively direct activity of disintegrins to the collagen receptor α1β1 integrin. Structurally, R/KTS-disintegrins are short, monomeric molecules containing 41 amino acids in its polypeptide chain [98]. The first KTS disintegrin was discovered in *Vipera lebetina obtusa* venom and named obtustatin [99]. Biological activities of disintegrins containing MLD- and KTS-motifs were investigated in many systems *in vitro* and *in vivo*. They are non-toxic in therapeutic doses in rodent and avian models, and their modulatory properties were observed in studies involving immuno-suppression of IDDM (insulin-dependent diabetes mellitus), asthma, neurodegenerative illness, cell apoptosis, as well as in cancer angiogenesis and metastasis [98].

1.5. Snake Disintegrins and Cancer

Disintegrins are capable of binding specifically to integrins, and studies exploring the potential of these molecules in interacting with integrins have inspired many studies, leading to the discovery of potential therapeutic agents. Additional, it has enabled a better understanding of processes involved in tumor development. In Table **1** we show the human integrins with their ligands, role in tumor growth and development and metastasis and their interaction with the snake venom derived disintegrins.

Jerdostatin, lebestatin, and obtustatin are three examples of disintegrins that bind to α1β1. Jerdostatin is a RTS recombinant that contains a disintegrin from *Protobothrops jerdonii*, which blocks the adhesion to collagens I and IV *in vitro* and angiogenesis *in vivo* [100]. Furthermore, r-jerdostatin inhibited the adhesion of rat aortic smooth muscle A7r5 cells (RASMCs) to immobilized CB3 fragment of collagen IV, causing retraction and detachment of cells. It also inhibited α1β1 integrin-dependent HUVEC tube formation, but did not affect the adhesion of human smooth muscle cells (SMCs) to CB3 fragment. Presumably, that happens because of the high expression of α2β1 integrin, which is compensated for α1β1 integrin blockage by jerdostatin. This scenario emphasizes the relevance of using specific inhibitors for analyzing the role of disintegrins in physiological and pathological conditions [101]. Lebestatin, in turn, is a member of KTS disintegrin family, and it is purified from Tunisian snake (*Macrovipera lebetina*) venom. Lebestatin interacts specifically with the α1β1 integrin, and it is able to inhibit both adhesion and migration of PC12 and α1β1 integrin-expressing CHO cells (CHO-alpha1) to type I and IV collagens. Additionally, this disintegrin affected adhesion and migration of EC and exhibited an anti-angiogenic effect *in vivo* [102]. Finally, obtustatin, the last example, is a disintegrin purified from the venom of the *Vipera lebetina obtuse*; it contains the sequence KTS in its active site loop. Obtustatin, as well as the integrins cited above, is a potent and selective inhibitor of α1β1 integrin and it does not inhibit the closely related integrin α2β1. It potently inhibited angiogenesis *in vivo* in the chicken chorioallantoic membrane assay, and it reduced tumor development by half in the Lewis lung syngeneic mouse model [99].

Rhodocetin, a RGD-containing peptide from *Callose-lasma rhodostoma*, acts as an α2β1 integrin inhibitor and antagonizes important cellular responses to type I collagen. Moreover, it prevents cell adhesion, migration, and collagen

Table 1. Preferential ligands, function on tumor formation and development and disintegrins interaction with human integrins.

Integrin		Ligand [26]	Function	Disintegrin
β1	α1	Collagen IV, laminin	Tumor growth, migration, invasion, angiogenesis	Viperistatin [154]
				Obtustatin [154]
				Jerdostatin [100]
				Lebestatin [102]
	α2	Collagen I, laminin	Tumor growth, angiogenesis, migration, invasion and intravasation, proliferation in the target tissue	Alternagin-c [155]
				Rhodocetin [103]
	α3	Laminin, fibronectin, TSP	Migration, invasion and intravasation, extravasation (adhesion to blood wall), proliferation in the target tissue	lebein-1 [156]
				lebein-2 [156]
	α4	VCAM-1, VEGF-A, OPN, Tenascin-C, angiostatin, tTG, factor XIII	Angiogenesis, migration, survival in the circulation, extravasation (adhesion to blood wall), proliferation in the target tissue	R-mojastin 1 [113]
				EO5 [105]
				VLO5 [105]
				EC3 [105, 157]
				EMS11 [95]
				Bitisgabonin-2 [158]
				Eristostatin [108]
	α5	Fibronectin, fibrinogen	Tumor growth, angiogenesis, migration, invasion and intravasation, extravasation	EMS11 [95]
				EMF10 [115]
				EO4 [95]
				VLO4 [95]
				VA6 [95]
				EC3 [105, 157]
				Cerastin [159]
				Lutosin [159]
				Crotatroxin [159]
				Durissin [159]
				Molossin [159]
				Viridin [159]
				Cereberin [159]
				Basilicin [159]
				Lachesin [159]
				Jararacin [159]
				Cotiarin [159]
				VB7 [95]
				Flavoridin [160, 161]
				Contortrostatin [125]
				Jarastatin [161]

(Table 1) contd….

Integrin		Ligand [26]	Function	Disintegrin
				Saxatilin [136]
				Bitisgabonin-1 [158]
				Vicrostatin [162]
				Ocellatusin [163]
				Rhodostomin(Kistrin) [88, 164]
				Cumanastatin 1 [165]
				Leberagin-C [122]
	α6	Laminin	Tumor growth, angiogenesis, migration, invasion and intravasation, extravasation, proliferation in the target tissue	Lebein-1 [156]
				Lebein-2 [156]
	α7	Laminin	Migration, invasion and intravasation	Lebein-1 [156]
				Lebein-2 [156]
	α8	Fibronectin, vitronectin, tenascin-C, OPN, and neph-ronectin	Tumor growth, angiogenesis, migration, survival in the circulation	Flavostatin [166]
				Elegantin [166, 167]
	α9	VCAM-1, VEGF-A, OPN, Tenascin-C, angiostatin, tTG, factor XIII	Tumor growth, angiogenesis, migration, invasion and intravasation	bitisgabonin-2 [158]
				VLO5 [105]
	αv	Fibronectin, vitronectin, fi-brinogen and osteopontin	Invasion and intravasation, migration	Saxatilin [136]
β2	αM	Fibrinogen, ICAMs, iC3b, factor-Xa	Tumor growth, survival in the circulation	Jarastatin [161]
β3	αv	Fibronectin, vitronectin, fi-brinogen, VWF, TSP, FGF-2	Tumor growth, angiogenesis, migration, invasion and intravasation, survival in the circulation, extravasation, proliferation in the target tissue	Accutin [168, 169]
				Accurhagin-C [170, 171]
				Contortrostatin [125]
				DisBa-01 [120]
				Echistatin [149]
				Insularin [172]
				Jarastatin [161]
				Leberagin-C [122]
				Rhodostomin (Kistrin) [88, 164]
				Cerastin [159]
				Lutosin [159]
				Crotatroxin [159]
				Durissin [159]
				Molossin [159]
				Viridin [159]
				Cereberin [159]
				Basilicin [159]
				Lachesin [159]

(Table 1) contd….

Integrin		Ligand [26]	Function	Disintegrin
				Jararacin [159]
				Cotiarin [159]
				Salmosin [117]
				Saxatilin [136]
				Flavoridin [160, 161]
				Triflavin [173]
				Trimestatin [174]
				Tergeminin [151]
				Eristicophin [151]
				Trigramin [88]
				Schistatin [175]
				Jerdonin [176]
				Vicrostatin [162]
	αIIb	Collagens, fibronectin, vitronectin, fibrinogen, VWF, TSP	Survival in the circulation, extravasation, adhesion to blood wall	Rhodostomin (Kistrin) [88, 164]
				Eristostatin [108]
				EC3 [105, 157]
				Contortrostatin [125]
				Barbourin [151, 177]
				Saxatilin [136]
				Echistatin [149]
				Bitistatin [178]
				Cerastin [159]
				Lutosin [159]
				Crotatroxin [159]
				Durissin [159]
				Molossin [159]
				Viridin [159]
				Cereberin [159]
				Basilicin [159]
				Lachesin [159]
				Jararacin [159]
				Cotiarin [159]
				DisBa-01 [120]
				Jarastatin [161]
				Schistatin [175]
				Insularin [172]
				Tergeminin [151]

(Table 1) contd….

Integrin		Ligand [26]	Function	Disintegrin
				Eristicophin [151]
				Triflavin [173]
				Elegantin [166, 167]
				Dendroaspin [179]
				Cumanastatin 1 [165]
				Albolabrin [180]
β5	αv	Vitronectin	Cell survival, angiogenesis, migration, invasion	Rhodostomin (Kistrin) [88, 164]
				R-mojastin 1 [113]
				Vicrostatin [162]
				Bitistatin [178]
				Saxatilin [136]
				Contortrostatin [125]
β6	αv	Fibronectin, tenascin	Cell proliferation, Migration, invasion and intravasation	Leberagin-C [122]
β7	α4	Fibronectin, VCAM, MAd-CAM	Proliferation in the target tissue	EC3 [105, 157]

lattice contraction *in vitro*. Studies reported that rhodocetin efficiently blocks cell invasion of HT1080 fibrosarcoma cells into a type I collagen matrix [103], delay tumor cell arrest, extravasation into the liver stroma, micrometastasis, and – although, it is not able to inhibit adhesion of liver-targeting tumor cells to the sinusoid wall components (laminin-1 and fibronectin), an essential step for liver metastasis – it remarkably blocked invasion *in vivo* [104].

Alternagin-C (ALT-C), a disintegrin-like protein purified from the venom of the Brazilian snake *Bothrops alternatus* also interacts with the major collagen I receptor, the α2β1 integrin. However, unlike rhodocetin, it has an ECD site. ALT-C inhibits the adhesion of a mouse fibroblast cell line (NIH-3T3) to collagen I, but when immobilized on plate wells, it supports the adhesion of this cell line, as well as of human vein endothelial cell (HUVEC). In addition, ALT-C induces HUVEC proliferation *in vitro* and *in vivo* angiogenesis, up-regulates the expression of 45 genes, including the VEGF gene, and down-regulates the expression of 30 genes, including VEGF gene and other growth factors leading to a proliferation effect. Moreover, it strongly activates Akt/PKB phosphorylation, a signaling event involved in endothelial survival and angiogenesis. In other words, ALT-C acts as a survival factor, promoting adhesion, endothelial cell proliferation and angiogenesis.

VLO5 (*Vipera lebetina obtuse*) and EO5 (Echis ocellatus) disintegrins express MLD and VGD motifs in their subunits and show a high degree of homology among themselves and other dimeric disintegrins. They proved to be potent inhibitors of α4β1 integrin [95]. Human (HS.939T) and mouse (B16) melanoma cell lines which express different integrins, including α4β1, adhered to immobilized VLO5 and EO5 [105]. VLO5 also completely abolishes vascularization induced by thrombospondin-1 (TSP-1) or its domain NoC1. Additionally, it showed a very potent anti-proliferative effect for dHMVEC. The ability of VLO5 to bind tumor cells, block endothelial cell proliferation and, as a consequence, angiogenesis – besides being related to α4β1 – appears to be linked to its interaction with α9β1 integrin which directly binds to VEGF-A, a potent inducer of angiogenesis and inducer of adhesion and migration of human endothelial cells [106, 107].

Eristostatin, isolated from *Eritocophis macmahoni* is another disintegrin that binds to α4β1 beyond αIIbβ3. It strongly inhibited lung and liver metastasis in a human melanoma experimental model in which B16F1 melanoma cells eristostatin-treated were injected in mice and efficiently inhibited adhesion of both MV3 and CHOa4 cells to α4β1-ligand VCAM-1 [108], [109]. Furthermore, it significantly impaired the migration of five human melanoma cell lines *in vitro* [110]. Cytotoxicity assays and direct binding assays using atomic force microscopy suggested that eristostatin acts by making the melanoma cells a better target for lysis by human natural killer cells [111].

R-mojastin 1, a RGD containing disintegrin cloned from the venom glands of the Mohave rattlesnake (*Crotalus scutulatus scutulatus*) that possibly recognizes α4β1 and αvβ5 integrins, inhibited platelet adhesion to fibronectin, ADP-induced platelet aggregation in whole blood and shows ability to inhibit platelet ATP release [112]. It also could be an useful tool in developing novel anti-tumor agents by its ability to inhibit tumor cell adhesion, migration and invasion *in vitro* [113].

Calvete and collaborators reported the isolation of a series of disintegrins from different venoms, which are able to bind to α5β1 integrin. Disintegrins VLO4 (*Vipera lebetina*

obtuse), VB7 (*V. berus*), VA6 (*V. ammodytes*), and EO4 (*Echis ocellatus*) displayed the RGD motif and prevented the adhesion of K562 cells, expressing the integrin α5β1 to immobilized fibronectin. On the other hand, disintegrin EMS11 (*Echis multisquamatus*) inhibited both α5β1 and α4β1 integrins with almost the same degree of specificity [95]. Although, VLO4 has shown no inhibitory effect on migration in endothelial cells [106], recently it was registered a patent of pharmaceutical compositions and methods for administering a combination of VLO4 with VP12 (an heterodimeric C-lectin type α2β1 antagonist) to be use in inhibiting, preventing or reversing angiogenesis, as well as in treating cancer [114]. EMF10, an heterodimeric disintegrin isolated from the *Eristocophis macmahoni* venom is an extremely potent and selective inhibitor of α5β1, being, therefore, a good candidate to be tested in antitumor assays [115]. Its sequence, disulphide-bond pattern, and molecular modelling were determined, but there are no studies about its activity.

Salmosin is a disintegrin containing the RGD sequence derived from the Korean snake venom (*Agkistrodon halys brevicaudus*) that binds to αvβ3 integrin, thus strongly inhibiting cell proliferation induced by basic fibroblast growth factor (bFGF), cell adhesion to ECM proteins, as well as cell invasion. This inhibitory action of salmosin may lead to cell cycle arrest and induction of active apoptosis. *In vivo*, it inhibits tumor-induced angiogenesis, interestingly without affecting the preexisting blood vessels or the angiogenesis that is critical for normal physiological processes. In addition, it showed a remarkable significant inhibitory effect on lung tumor colonization in B16F10 melanoma experimental metastasis [116, 117]. Subsequent studies suggest that suppression of tumor cell growth occurs by specifically inhibiting the αv subunit and that the salmosin's mechanism of inhibition, tested in bovine capillary endothelial (BCE) cells, seems to be related with disassemble of cortical actins at focal adhesions and cell induction to be rounded and detached, but without alter microtubule structures in the early stage of cells. This study showed that salmosin inactivated FAK-dependent integrin signaling pathways, since in salmosin-treated BCE cells, focal adhesion kinase (FAK) was dephosphorylated and expression of paxillin and $p130^{CAS}$ decreased, but PI3 kinase, ILK, and β-catenin's expressions levels did not decrease [118, 119].

DisBa-01, a recombinant RGD disintegrin isolated from a cDNA library made with RNAs from the venom gland of *Bothrops alternatus* snake venom, also blocks αvβ3 integrin by binding to vitronectin. However, *in silico* model suggests that DisBa-01 should recognize the other aIIbb3. DisBa-01 inhibits cell migration, besides having anti-angiogenic and anti-metastatic properties *in vivo*. Furthermore, it does not affect the binding nor the proliferation of a human breast cancer-derived cell line (MDA-MB-231), not expressing αvβ3 [120]. The mechanism of action of DisBa-01 was investigated and the results showed that it might induce distinct effects in the cells of the tumor microenvironment. It strongly decreases the expression of VEGF mRNA and of its receptors, VEGFR1 and VEGFR2 in endothelial cells, and at nanomolar concentrations also modulates the activity of MMP-2 and MMP-9 [121].

A notable exception of a disintegrin that interacts with αvβ3 is Leberagin-C, a member of the disintegrin-like/cysteine-rich family. It was purified from the venom of Tunisian snake *Macrovipera lebetina transmediterranea* and has a SECD motif in its disintegrin-like domain. Leberagin-C may interact with αvβ3 and, to a lesser extent, with αvβ6 and α5β1 integrins. This disintegrin is able to prevent platelet aggregation induced by thrombin and arachidonic acid, the adhesion of melanoma tumor cells on fibrinogen and fibronectin [122].

Contortrostatin (CN), a RGD disintegrin from southern copperhead (*Agkistrodon contortrix contortrix*) snake venom is one of the most studied snake disintegrins. It binds to integrin αvβ3, but is also able to recognize αIIbβ3, α5β1, and αvβ5 integrins, thus having a wide variety of complex effects. Contortrostatin was first purified by Trikha, Rote, & Manley in 1994 [123]. Since then, other studies have been developed to characterize its biological effects and mechanisms of action both *in vitro* as *in vivo* [124-126]. CN is a potent inhibitor of cell adhesion, migration, and angiogenesis *in vitro*. *In vivo*, it inhibits tumor growth, neovascularization, and metastasis [127, 128]. In an orthotopic xenograft model, local injection of contortrostatin into human breast cancer (MDA-MB-435) tumor masses inhibited its growth by 74% and reduced the number of pulmonary macro and micrometastasis by 68 and 62.4%, respectively. In this case, CN was not cytotoxic to cancer cells, and did not inhibit proliferation of the breast cancer cells *in vitro*. However, it inhibited angiogenesis, thus preventing tumor progression [125, 129, 130]. In spite of all these activities *in vitro* or *in vivo*, CN directly did not affect cell viability or MMP-2 and MMP-9 activity. It may induce apoptosis in an anchorage-dependent mechanism, disrupting actin cytoskeleton and altering the distribution at intercellular contacts of VE-cadherin. Moreover, contortrostatin downregulates FAK and paxillin tyrosine phosphorylation, which may be crucial for actin cytoskeleton disruption, leading to inhibition of cell proliferation and invasion [126, 131]. Other snake venom disintegrins have been identified with similar activities. Most of them have the RGD sequence, such as accutin from *Agkistrodon acutus* and triflavin from *Trimeresurus flavoviridis* that binds to αvβ3 and αIIβ3 or rhodostomin (also known as kistrin) from *Calloselasma rhodostoma*; this last one binds to αvβ3, α IIbβ3, and α vβ5. Leberagin-C from *Macrovipera lebetina*, however, unlike others already mentioned, shows a SECD site and binds to αvβ3, and, to a lesser extent, αvβ6, and α5β1 integrins.

Disintegrins able to block αIIbβ3 has also shown promising and interesting activities. Saxatilin, for example, an RGD containing disintegrin from *Gloydius saxatilis*, inhibits collagen-induced platelet activation, thereby suppressing platelet granular secretion, as well as subsequent endothelial cell migration and invasion [132]. It significantly inhibited cancer cell invasion induced by tumor necrosis factor-alpha (TNF-α) and reduced MMP-9 mRNA levels in MDAH 2774 human ovarian cancer cell line [133]. Furthermore, saxatilin also inhibited VEGF production, suppressing the angiogenesis-inducing properties of NCI-H460 human lung cancer cells. This occurs by affecting hypoxia induced factor-1α (HIF-1α) expression via the Akt pathway [134]. *In vivo* expression of saxatilin was able to strongly inhibit tumor

growth by preventing endothelial cell proliferation and smooth muscle cell migration. However, its antitumor efficacy individually expressed *in vivo* was not sufficiently potent to lead to tumor regression *in vivo*. Thus, combinational transfer of saxatilin together with angiostatin and endostatin (fragments of plasminogen and collagen respectively of naturally-occurring and known as inhibitor of tumor growth) genes resulted in the most effective inhibition of angiogenesis, induced inhibition of B16BL6 melanoma growth, and pulmonary metastasis. Treatment with the three plasmids reduced B16BL6 tumor growth by 89% and pulmonary metastasis by 90%, compared with the empty vector-treated control group [135]. Integrin-binding assays showed that in addition to inhibit αIIbβ3, saxatilin also binds to α 2bβ3, α5β1, α vβ3, α vβ1, and α vβ5 [136]. This methodology provides additional evidences supporting this approach as an alternative procedure for antiangiogenic cancer therapy.

1.6. Molecular Imaging

Theranostics is a term coined for the study of drugs or methods used for simultaneous diagnosis and treatment, usually involving nanocapsules, bubbles, particles or tubes that has focused mainly in cancer research during the last years [137], since cancer is one of the most dangerous diseases nowadays. As discussed previously, snake-derived disintegrins are able to identify diverse cancer cells and biological processes, becoming a promising molecule in which theranostic studies may be done.

Leong-Poi and colleagues [138] demonstrated that echistatin-conjugated microbubbles (MBE) exhibited a high potential to image activated endothelium in subcutaneously implanted matrigel plugs enriched with FGF-2. Microbubble retention inside the microvasculature was much higher as compared to non-targeted microbubbles, showing that echistatin can be used for targeted imaging approaches. Another study using echistatin targeting microbubbles as a binding ligand to image αvβ3 expression in human glioma cells implanted intracerebrally in rats showed a significant retention within tumors, and also reported increasing accumulation after 14 days of tumor growth [139].

The integrin receptor αvβ3, as mentioned previously, has been explored as a marker for tumor angiogenesis, since it is found on endothelial cells lining new growing blood vessels at a higher density than on mature blood vessels. Bitistatin, a disintegrin originally isolated from the venom of the puff adder *Bitis arietans*, has affinity to both αIIbβ3 and α vβ3 integrins. It can be radiolabeled, injected systemically and then detected, demonstrating to be a promising agent for *in vivo* molecular imaging this approach was done successfully to diagnose thrombosis in a canine model [140]. Currently, recombinant bitistatin labeled ^{99m}Tc-HyNic-rBitistatin has been studied in human subjects in a Phase II clinical trial (ClinicalTrials.gov Identifier: NCT00808626). When labeled with ^{125}I or with ^{64}Cu, bitistain was shown to accumulate in tumors even in non-expressing αvβ3 integrin, suggesting to be mediated by a combination of αvβ3 and αIIbβ3 integrins [141]. Other known snake disintegrins were also labeled for example, as echistatin (^{125}I labeled) and eristostatin (FITC-labeled), and analysis in flow cytometry showed no alteration of disintegrins' biologic activity [142, 143].

1.7. Common Problems in the Clinical Application of Disintegrins

The complexity of cancer, which involves different integrins, often makes it difficult to identify the exact function of each integrin in tumor development. On the other hand, this diversity opens an ample scope for the use of specific inhibitors and with fewer side effects, differently from most current anticancer therapies (radiotherapy, chemotherapy) that are not specific and target both tumor and healthy cells. Thus, in recent years, new treatments tend to focus on the specific tumor microenvironment and particularly on the inhibition of tumor angiogenesis [144]. Although, disintegrins are highly effective in binding and inhibiting integrin function, which draws attention to its therapeutic potential, most clinical studies have not progressed beyond early clinical development due to the problems of instability and immunogenicity, common to peptide-based drugs. Furthermore, natural products are typically a limited source of supplies, which require the development of methods for synthetic production or heterologous expression.

In order to reduce the instability and immunogenicity of disintegrins, efforts have been undertaken to develop new approaches. As mentioned previously, daily administration of salmosin is able to suppress tumor progression. However, it is very difficult to maintain its therapeutic levels in the blood by systemic administration. Therefore, Kim and colleagues developed a unique lipoplex method for delivering salmosin DNA *in vivo*, where salmosin gene is administered with cationic liposomes. Subcutaneous administration of the salmosin gene resulted in systemic expression and concomitant inhibition of the growth of B16BL6 melanoma cells and suppression of pulmonary metastases. These results suggest that the administration of the salmosin gene complexed to cationic liposomes is effective in maintaining salmosin at an effective therapeutic level and may be clinically applicable to anticancer gene therapy. Nonviral gene delivery using complexes of cationic liposomes suggest administration of this kind of research into cancer gene therapy. As demonstrated, liposomal vectors provide salmosin gene with effectiveness and maintenance of distinct advantages over recombinant viral vectors, because they are able to supply antiangiogenic salmosin at an effective therapeutic level, being clinically nonpathogenic, less immunogenic, simple to prepare, and applicable to anticancer gene therapy [145, 146].

The antitumor activity of contortrostatin (CN), earlier mentioned, was described in a mouse model of human mammary cancer, and the method of delivery was daily intratumor injection. This alternative is not translatable to clinical application, so Swenson and coleagues developed a clinically relevant method of administering using liposomal delivery. The advantages of liposomal delivery of CN are that it has a significantly prolonged circulatory half-life compared with native protein, is passively accumulated in the tumor, has no platelet reactivity, and is not recognized by the immune system. Regarding to biological activity, it was completely preserved, leading to potent antiangiogenic activity in the orthotopic xenograft human mammary tumor model [147]. Afterwards, a similar liposomal formulation was used and the preparation also showed to provide effective *in vivo* antitumor and anti-angiogenic activity in a human ovarian cancer

animal model [125]. In another research, the same liposomal encapsulation procedure was applied, but instead of native CN, they used a recombinant protein. This study showed that CN can be easily and cost-effectively produced recombinantly and shows excellent anti-tumor efficacy in the breast carcinoma model [148]. Recombinant DNA technology has been employed successfully to produce large quantities of proteins as the disintegrins, since snake venoms are an impractical source of proteins for clinical application.

Some antibodies have been extensively used with similar applications to disintegrins, but to use disintegrins regarding to antibodies is more advantageous for several reasons, among them: disintegrins have a shorter half-life, are susceptible to inhibition, easier to control, less immunogenic, have a lower cost, and do not show availability problems that antibodies have [138].

1.8. Drugs in Clinical Tests

Although so far no drug has been produced from a native molecule purified from venom, several peptidomimetics were designed by basing on the structure of these molecules. Two drugs, have been designed based on snake venom disintegrins and are available in the market as antiplatelet agents: Tirofiban (Aggrastat®) and eptifibatide (Integrilin®), (reviewed by Koh and Kini, 2012). Hence, these drugs opened the door to the development of novel and potent therapies to many types of sickness.

Aggrastat® (tirofiban) is based on the distance separating the side chains of Arg and Asp in the RGD motif of echistatin, a disintegrin from *Echis carinatus*, wich binds to αIIbβ3 and αvβ3. In addition to that, it is a GPIIb-IIIa inhibitor and promotes platelet adhesion and protein tyrosine phosphorylation. The FDA approval for anticoagulant use was obtained in 1998, and it has been used to myocardial infarct and refractory ischemia [149, 150].

Integrilin® (Eptifibatide) has even closer links to snake venom disintegrins. In order to discover a disintegrin specific for αIIbβ3, 62 snake venoms were screened, leading to the identification of barbourin, a disintegrin from *Sisturus miliarus barbouri* with KGD site that binds to αIIb3 integrin. The substitution of Lys to Arg lead to the increase specificity for αIIbβ3, so using this knowledge, pharmacophore designed and synthesized integrilin that received FDA approval for use in acute coronary syndromes [151, 152].

CONCLUSION

Since disintegrins were first discovered, it was immediately appreciated that these small naturally-occurring proteins had significant promise as tools in the analysis of cell biochemistry and function. In addition, disintegrins from snake venoms appear to be very useful tools in pharmacology, in the development of new pharmaceuticals and in helping to understand mechanisms related to cancer. As integrins are intimately involved in cancer cell survival, motility, invasion, angiogenesis, and other processes critical to block cancer establishing, progression, invasion, differentiation and metastasis, disintegrins are a very useful platform to develop new drugs to cancer treatment. Moreover, they may be used not only as a pattern for developing new therapeutics to control cancer, but may be investigated as components to early detection or even on therapeutic monitoring. Therefore, research involving snake venom disintegrins appears to have a very wide application in medicine. A summary of the specific interaction of disintegrins with integrins expressed on various cell types was illustrated here, demonstrating the potential use of disintegrins for diagnosis, screening and treatment of cancer.

Results obtained so far are exciting, although many molecules did not advance to clinical trials. However, eventually some of them will be successful, as has already happened with some, not in cancer biology, but in other areas related to hemostasis. This initial scenario may be the case, because the study of disintegrins in relation to cancer is relatively recent and around only 10% of venoms from all snakes from the Atractaspididae, Elapidae,Viperidae and Colubridae families have been analyzed for the presence of disintegrin genes, mRNAs or proteins [153]. Therefore, it is certain that those molecules remain a fertile area of study.

LIST OF ABBREVIATIONS

bFGF	=	Basic fibroblast growth factor
BMDCs	=	Bone marrow derived cells
CLCA	=	Chloride channel calcium-activated
ECM	=	Extracellular matrix
EMT	=	Epithelial-mesenchymal transition
FGF	=	Fibroblast growth factor
FGFR	=	Fibroblast growth factor receptor
FLIP	=	Flice-like inhibitory protein
HIF	=	Hypoxia induced factor
MMP	=	Matrix metalloproteinase
MP	=	Metalloproteinase
NF-κB	=	Nuclear factor-κB
OPN	=	Osteopontin
PI3K	=	Phosphatidylinositol 3-kinase
SVMP	=	Snake venom metalloprotease
TNF-α	=	Tumor necrosis factor-alpha
tTG	=	Tissue-type transglutaminase
VCAM	=	Vascular cell adhesion molecule
VEGF	=	Vascular endothelial growth factor
VEGFR	=	Vascular endothelial growth factor receptor
VWF	=	Von Willebrand factor

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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